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Pesticides and Microbial Degradation in the Subsurface Sediments in Finland

PESTICIDES AND MICROBIAL DEGRADATION IN THE SUBSURFACE SEDIMENTS IN FINLAND

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Tiivistelmä Referat – Abstract

Three target pesticides in this study were atrazine, simazine, and BAM (2,6 – dichlorobenzamide). Atrazine and simazine have been used in the weed control world widely for over 50 years. The chlorine in triazine ring in their molecular structures is known to be resistant to degradation under the aerobic condition. BAM is the main persistent metabolite from the degradation of pesticide dichlobenil. Compared with dichlobenil, BAM is very mobile, and easily leached to the groundwater. The main goals of this study were to investigate 1) where in sediments the three pesticides are accumulated in Lahti, 2) the potential of microbes and carbon source supplementation in enhancing the degradation of atrazine or BAM, and 3) the isolation and characterization of microbes capable of degrading atrazine or BAM.

In August 2005, the sediment samples used were taken from fields with long-period-pesticide use history in southern Finland. Atrazine, simazine, and BAM were extracted by adding methanol : H_2O (3:1 v/v) as the extraction solution before analyzing by HPLC (High Performance Liquid Chromatography). The added internal standard, together with a series of standard solutions were used to calculate the pesticide concentrations in the subsurface sediments. In the second part of this study, three experiments were built up to monitor the biodegradation of atrazine and BAM, which were, atrazine degradation in soil column with the continuous water circulation, and atrazine and BAM degradation in soil suspensions using shaker flasks. The cultivated microbes (e.g. *Pseudomonas* ADP, *Zoogloea* sp. K-1A2), sodium citrate, and atrazine/BAM were added as the microbial degraders, the carbon source, and the nitrogen source into bioreactors, respectively, to stimulate the microbial degradation of pesticides. Daily, 2 * 100 µl of liquid samples were taken for the HPLC analysis and microbial cultivation, to follow the pesticide degradation and microbial growth. From pure cultures of the bacterial and fungal colonies with different characteristic visible features, DNA isolation was done, following by the amplification by PCR (Polymerase Chain Reaction), and the sequencing analysis, to identify the pesticide degraders.

As expected, atrazine, simazine, and BAM were detected in the subsurface sediments in Lahti, Finland. The concentrations of pesticides varied between 5.3 ± 0.5 – 189.6 ± 92.1 ng/g dw in railway station, and 5.34 ± 0.2 - 34.78 ± 6.4 ng/g dw in Lahti city garden. All three pesticides reached their highest concentrations when the sediment type was clay, and their concentrations dropped significantly close to the water table.

In almost all the treatments, atrazine / BAM concentration dropped rapidly during the first day, thereafter, it remained at about the same level until the end of experiment. The fastest degradation was found in the treatment with *Pseudomonas* ADP / *Zoogloea* sp. K-1A2 supplementation. Sodium citrate stimulated the microbial growth but not the degradation of atrazine / BAM. Sediments likely contained microbes able to use atrazine / BAM as the nitrogen source, due to the found degradation in the control treatments. Much more atrazine was degraded in soil columns with water circulation than in soil suspensions. In the end of the study, the sequencing analysis brought up a few strains which were not recognized as the degraders before.

Avainsanat – Nyckelord – Keywords
atrazine, BAM, degradation, *Pseudomonas* ADP, *Zoogloea* sp. K-1A2,
soil column, water circulation, soil suspensions

Säilytyspaikka – Förvaringställe – Where deposited
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Muita tietoja – Övriga uppgifter – Additional information

1. INTRODUCTION

Pesticides comprise the largest group of xenobiotic compounds introduced into the environment. The compounds are used for controlling weeds, insects, and fungi in agriculture, orchards, and public areas world widely. During the last decades there has been an increased frequency of pesticide detection in ground and surface water in Europe (European Environment Agency, 1999) and the United States (Barbash *et al.,* 2001). The use of potentially harmful or toxic pesticides is regulated in European Union. The EU threshold concentration for one pesticide in drinking water is 0.10 µg/l and for several pesticides 0.50 µg/l (Holtze *et al.,* 2006). The knowledge of the impacts of these molecules on complex environmental conditions is not complete. Pesticides enter the surface and ground water mainly as a run-off from agricultural fields, and they are usually found from the waters near agricultural areas. The contamination of groundwater by pesticides occurs often when the treated area is susceptible to the leaching (Navarro *et al.,* 2006). Nowadays, growing attentions have been given to the persistent metabolites of pesticides, produced due to the partial degradation (Boxall *et al.,* 2004; Sinclair and Boxall, 2003). Some metabolites in soil are more mobile and less susceptible to biodegradation than their mother compounds and as a consequence they are detected more frequently in nearby groundwater resources (Holtze *et al.,* 2007). One example is the metabolite BAM (2,6-dichlorobenzamide) originating from degradation of the herbicide dichlobenil (2,6-dichlorobenzonitrile). The target compounds in this study are atrazine, simazine, and the degradation product of dichlobenil, BAM.

1.1 s-Triazines

s-Triazines pesticides, simazine (2-chloro-4,6-bis [ethylamino]-*s*-triazine) and atrazine (2-chloro-4-ethylamine-6-isopropylamino-*s*- triazine) are very similar in structures (Fig. 1). They have been used world widely in the weed control for over 50 years (Ertunç *et al.*, 2002; Liu *et al.*, 2003; Ralebitso *et al.*, 2002). After application, atrazine and simazine are readily absorbed by the plant roots (Crafts 1965) and spread rapidly to the top of the weed seedlings. They interfere with the enzyme system responsible for the photolysis of water and halt the process of photosynthesis (Mandelbaum *et al.*, 1993a; Ralebitso *et al.*, 2002, Martin-Laurent *et al.*, 2003; Ashton & Klingman, 1982; Dean *et al.*, 1996). Atrazine has been found to be toxic to animals and is classified as Class C or potential human carcinogen. Its dealkylation metabolites also are regulated compounds and may pose health risks to vertebrates (Ralebitso *et al.*, 2002).

Compared with simazine, atrazine is used more widely in the world. Since being introduced in the 1950's, atrazine has been used very commonly in agriculture, forestry, industrial sites, and railroad embankments for controlling broadleaf weeds (Graymore *et al.*, 2001; Tappe *et al.*, 2002; Dean *et al.*, 1996). Thus, these two xenobiotic compounds are both among the most widely applied pesticides in the USA, where 38,000 tons of atrazine and simazine were purchased during 2001 (U.S.EPA, 2004). In Finland, the sale of atrazine has been banned since 1992. At least partly simazine has been replacing atrazine use until the year 2002, when the sale of simazine also was banned. Although both atrazine and simazine have not been used for many years in Finland, it doesn't mean that they have despaired from our life. The sied branches in the s-triazine ring molecular structure of atrazine and simazine makes them resistant to degradation. Nowadays, atrazine is still in use in many agricultural countries like USA, India, and China, and simazine is at least still

in use in desert and golf course in USA.

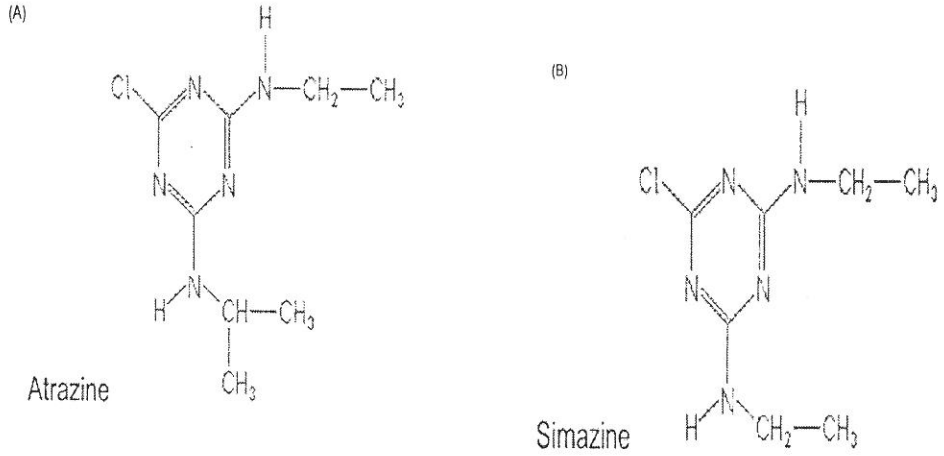


Figure 1. Structural formulas of atrazine (A) and simazine (B)

1.1.1 Atrazine in the environment

Atrazine is moderately persistent in the environment with a half life of 4 to 100 days, and its decomposition products are also known to be relatively persistent (Mandelbaum *et al.,* 1993a, b). After the application, the amount of atrazine bound to soil particles depends on several variables, including the soil structure, organic matter content, moisture content, clay percentage, soil pH, and particle size distribution (Dean *et al.,* 1996). Atrazine tends to absorb to humic substances, especially to humic acids, clays and oxyhydroxides (Ralebitso *et al.,* 2002). It has been reported that atrazine easily leaches into groundwater and causes contamination (Mandelbaum *et al.,* 1993b, Ralebitso *et al.,* 2002) due to its moderate hydrophilicity and high aqueous solubility of 33 mg/l. Atrazine persists under cool, dry conditions, and in a stable pH environment (Graymore *et al.,* 2001).

The atrazine molecule has been resistant to degradation under aerobic conditions (Jessee *et al.,* 1983) which greatly increases the chance for long-term environmental contamination. When Cl- is removed from atrazine, the molecule is regarded as harmless. Generally, atrazine can be degraded under aerobic conditions in a limited situation, but likely not always. The development of a safe and economical clean-up technique for atrazine is needed. There are several known methods to remove atrazine from soil, like land removal, incineration, ozonation, photochemical degradation, diatomaceous earth remediation, powdered activated carbon absorption and biological remediation (Ralebisto *et al.,* 2002, Wackett *et al.,* 2002). In contrast to other alternatives, bioremediation method has the minimal environmental impacts for the detoxification of environmentally hazardous chemicals.

1.1.2 The degradation products of atrazine

Atrazine may degrade into many metabolites, each of varying persistence and toxicity. The most common metabolites of atrazine are hydroxyatrazine (HA), desethylatrazine (DEA), deisopropylatrazine (DIA), desethyldeisopropylatrazine (DEDIA), deisopropylhydroxyatrazine (DIHA), and desethylhydroxyatrazine (DEHA). The processes that affect the fate of atrazine in soil include chemical degradation (i.e. adsorption, volatilisation, photodegradation), and microbial degradation. DIA, DEA, and DEDIA are formed through biodegradation, whilst HA and DEHA can be formed by either chemical reactions in the soil or biodegradation. Like in soils, the degradation of atrazine in water results from both biological and chemical processes. Bacteria and fungi split ethyl groups from the triazine ring for energy, whereas chemical hydrolysis releases chloride ions. Chloride ions can be released also by microbiological activity.

1.1.3 The microbial degraders of atrazine

There are two biochemical pathways for microbes to degrade atrazine, plasmid-bound genes and mono-oxygenase related aerobic degradation. The microbial degradation depends on the amount of available organic matter within the soil. In the case of atrazine degradation, the bacteria catabolise atrazine mostly as a nitrogen source while an additional carbon, likely sodium citrate makes the catabolism more effective (Mandelbaum *et al.,* 1995; Ralebisto *et al.,* 2002; Wackett *et al.,* 2002). Researchers have proved that bacteria or mixed cultures that could use atrazine as the sole C source (Behki and Khan 1986; Yanzekontchou and Gschwind 1994) or as the sole N source (Assaf and Turco 1994; Alvey and Crowley 1995; Mandelbaum *et al.,* 1995; Radosevich *et al.,* 1997).

The hydrolytic reactions of atrazine degradation were first identified in *Pseudomonas* sp. strain ADP. The genes that encode the enzymes are located on a large self-transmissible plasmid. When examining *Pseudomonas* ADP, scientists have found the atrazine degrading operon *atz*, which has the genes *atz*A, *atz*B, *atz*C, *atz*D, *atz*E and *atz*F (de Souza et al., 1996; Boundy-Mills *et al.,* 1997; Sadowsky *et al.,* 1998; Martinez *et al.,* 2001; Wackett *et al.,* 2002; Martin-Laurent *et al.,* 2003; Devers *et al.,* 2004). Similar genes have also been found in *Chelatobacter heintzii*, *Rhizobium* strain PATR, *Alcaligenes* SGI, *Agrobacterium radiobacter* J14a, *Ralstonia picketti* D and *Clavibacter michiganensis* ATZ1 (Seffernick *et al.,* 2000, Devers *et al.,* 2004).

1.1.4 Factors affecting atrazine degradation

Atrazine is easily accumulated in soil after application and its degradation is either a microbial or chemical process, under aerobic or anaerobic conditions (Blumhorst and Weber 1994; Assaf and Turco 1994; Mandelbaum *et al.,* 1995; Accinelli *et al.,* 2001). There are many factors which would affect the degradation of atrazine in soil, like the soil type, depth, moisture conditions, temperature, oxygen amount, pH, and organic matter (Houot *et al.,* 1999; Jenkins *et al.,* 1997; Kruger *et al.,* 1993). The cold temperature and low microbial activity in groundwater cause pesticide degradation slower than at the soil surface (Graymore *et al.,* 2001).

1.2 BAM (2,6 – Dichlorobenzamide)

Dichlobenil is used world widely for total weed protection in orchards, ornamental plants and on nonagricultural sites (courtyards, driveways, parking areas, railroads, etc.) (*Clausen et al.,* 2004). In soil, the triple-bond of dichlobenil is turned to double-bond, and then the persistent and very mobile metabolite BAM is formed. Data have shown that the sorption of BAM is minor in both soil (Clausen *et al.,* 2004; Holtze, 2005) and subsurface sediments (Broholm *et al.,* 2001; Clausen *et al.,* 2004; Tuxen *et al.,* 2000). The organic carbon-normalised sorption coefficient, Koc values of 33-35 $l\,kg^{-1}$ indicate that BAM is easily leached to groundwater and may cause problems (Holtze *et al.,* 2007). Dichlobenil was detected in 0.9% of the Danish drinking water wells in 2003, and the concentration exceeded the EC threshold value in 0.05% of the wells. The detection of dichlobenil is relatively rare compared to the detection of its metabolite BAM which was found in 20.5% of the wells in the period 1992-2002 (Geological Survey of Denmark and

Greenland, 2004). Although dichlobenil is commonly degraded to BAM, the further degradation of the BAM intermediate could not easily be demonstrated, due to the chloro-substituents in the structure (Holtze *et al.*, 2006). World widely, BAM has been detected in groundwater in Finland, Denmark, Germany, Italy, the Netherland, and Sweden (Kreuger *et al.*, 2003; Porazzi *et al.*, 2005; Versteegh and te Biesebeek, 2003).

Denmark is the only country which has banned the use of dichlobenil since 1997. In 2006 BAM was still the most frequently encountered groundwater contaminant in this country (Holtze *et al.*, 2007). In Finland, dichlobenil is still in use although the sale has decreased year by year, and the use of dichlobenil is forbidden in the groundwater area. For example, the sale of dichlobenil was 5040 kg in 2006, which was 760 kg less than in 2005 (Evira, 2006). In the United States 110-161 tons of dichlobenil was used during 1993-1995 (U.S.EPA, 1998) and in Denmark 556 tons was applied from 1970 to 1996 at a typical dose of 4-30 kg ha^{-1} (Elkjær *et al.*, 2002).

(A) (B)

Figure 2. Structural formulas of dichlobenil (A) and BAM (B)

1.2.1 The toxicity of BAM and dichlobenil

The acute toxicity of BAM is considered to be low to moderate (Holtze *et al.*, 2007). Based on all available toxicity data, the US EPA made a conclusion that dichlobenil or BAM is not an acute dietary risk in drinking water in ecologically relevant concentrations (U.S. EPA, 1998). The pesticide residue concentrations detected in groundwater are normally in the range of ng-mg per l^{-1} (Geological Survey of Denmark and Greenland, 2004), which is significantly lower than any levels measured to have toxic effects in the literature. However, it should be kept in mind that the carcinogenic and endocrine disrupting affects caused by long-time exposure to low BAM concentrations in drinking water are still unknown.

1.2.2 The microbial degradation of BAM

There has not been published many studies on BAM degradation. The two first BAM-mineralising bacteria, *Aminobacter sp.* strains ASI1 and MSH1, have been isolated from two different dichlobenil-exposed soils. Both degraders are capable of using BAM as a source of both carbon and nitrogen (Simonsen *et al.*, 2006; Sørensen *et al.*, 2007). The genus *Aminobacter* is ubiquitous in natural environments (Rousseaux *et al.*, 2001) and characterised by facultative methylotrophy and methylamine utilisation (Urakami *et al.*, 1992). Pesticide-degrading bacteria belonging to this genus have previously been isolated from soil, including atrazine-degrading strains (Rousseaux *et al.*, 2001; Topp *et al.*, 2000).

1.2.3 Factors affecting BAM degradation

The molecular structure and sorption are both rather important factors related to the degradation of BAM. The other factors affecting the degradation include soil pH, total organic carbon, temperature, humidity, clay or calcium carbonate contents (Clausen *et al.*, 2006; Nimmo and Verloop, 1975; Verloop, 1972). However, no clear correlations between these factors and BAM degradation have been established.

1.3 Aims of this study

Atrazine, simazine, and BAM were the three target pesticides in this study, and at least atrazine and BAM have been detected in the groundwater in Finland. The interests of the study were to investigate

1) Where in sediments the three pesticides were accumulated in Lahti,
2) Whether the atrazine and BAM degradation can be enhanced by adding carbon source or the known degrading microbes,
3) Isolate and characterize microbes able to degrade atrazine or BAM.

The hypothesis was that atrazine, simazine, and BAM will be detected in the subsurface sediments. In the bioreactor experiments, the hypothesis was that the supplemented carbon source and degrading microbes help the degradation of atrazine and BAM; and some microbial degraders will be found in the end of the experiment.

2. MATERIALS AND METHODS

2.1 Sampling

The samples were collected for determining (i) the efficiency of pesticide extraction, (ii) the amount of pesticides in sediments in the area where pesticides are known to be used and groundwater contained pesticides, and (iii) the microbial degradation efficiency of atrazine and BAM in subsurface sediments. The dry weights (dw) of all samples were measured after drying in oven at 105 °C for 24 hours from three replicates.

2.1.1 Pre-experimental sample

In order to test the efficiency of the pesticide extraction, the pre-experimental surface sample (0 - 10 cm) was taken from a garden with no previous pesticide history near the Department of Ecological and Environmental Sciences, University of Helsinki in Lahti, Finland. The collected samples were stored in sterilized plastic bags, and transported to the laboratory. Three replicates of 60 g dw sample were placed in sterilized Erlenmeyer flasks with the supplementation of 1 μg/g dw of analytical grade atrazine, simazine, BAM (99.1 %, Dr Ehrenstorfer GmbH, Augsburg, Germany). Soil and pesticides were mixed carefully with sterilized glass rod, and incubated in shaker (200 rpm) for 4 days in dark at 21 ± 2 °C. The sample with supplemented pesticides was divided into three 50 ml Falcon tubes, 10 g dw per tube, and frozen at -20 °C. The pesticides were extracted to determine the extraction efficiency.

2.1.2 Pesticides in subsurface sediments

Sediments for studying the pesticide concentration in subsurface soil were collected during August 15-19, 2005 in areas where pesticides are known to be used to control the grass growth. Two sediment sampling places in Lahti were railway station area and Lahti city garden located above the Laune groundwater area (Fig. 3). The samples were taken with the drilling equipment (Fig. 4) from soil surface down to the depth of 33 m in Lahti city garden, and 55 m in the railway station area. The groundwater table was 15.0 m in railway, and 4.6 m in Lahti garden. The diameter of the drill was ø 75 mm in the depths of 0-10 m in Lahti garden and between 0-20 m in railway station. The drill with the diameter of ø 48 mm was used below 10 m depth in Lahti garden and below 20 m depth in railway station. The sediments were removed from the drill (Fig. 5), collected to plastic bags, and then transported to the laboratory. Six samples were taken from different depths to measure pesticides in these two sampling places (Table 1). The samples were from above and below the groundwater table.

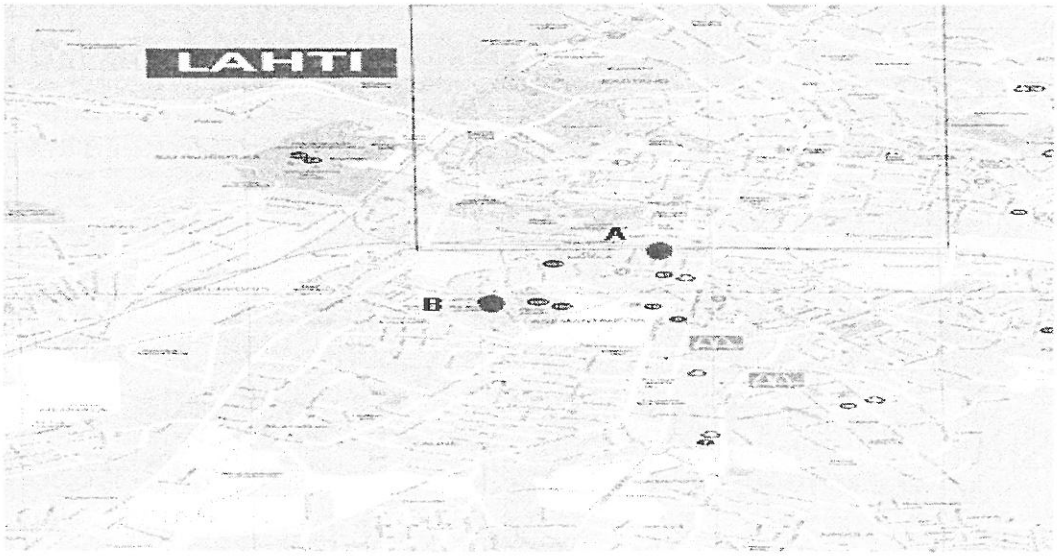


Figure 3. Sampling places in Lahti were railway (A) and Lahti Garden (B).

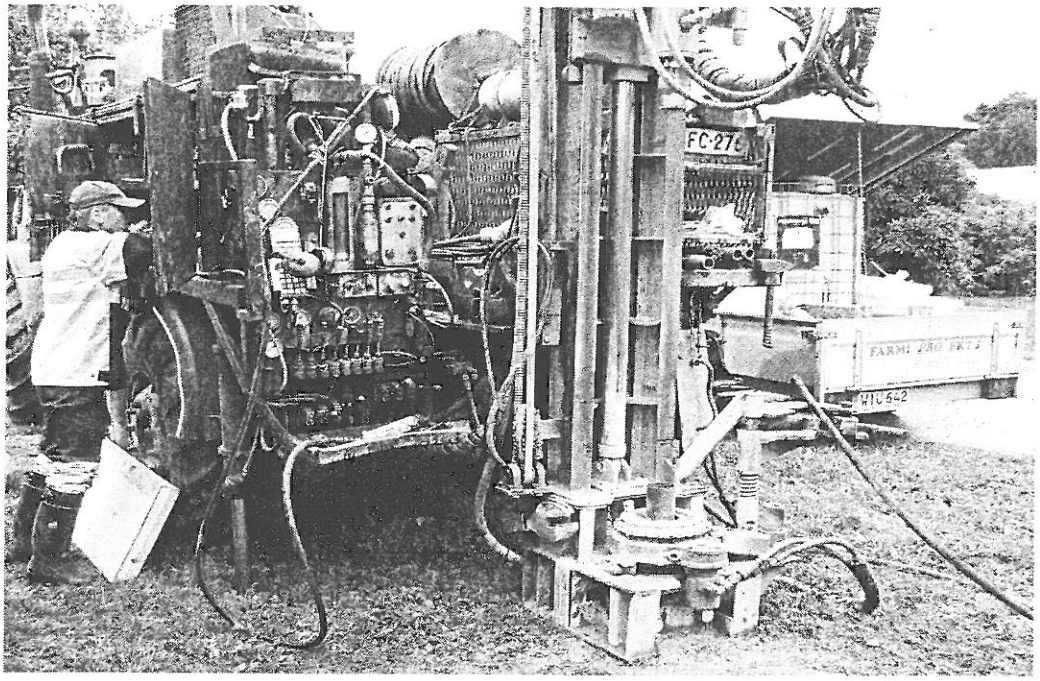


Figure 4. The drilling equipment.

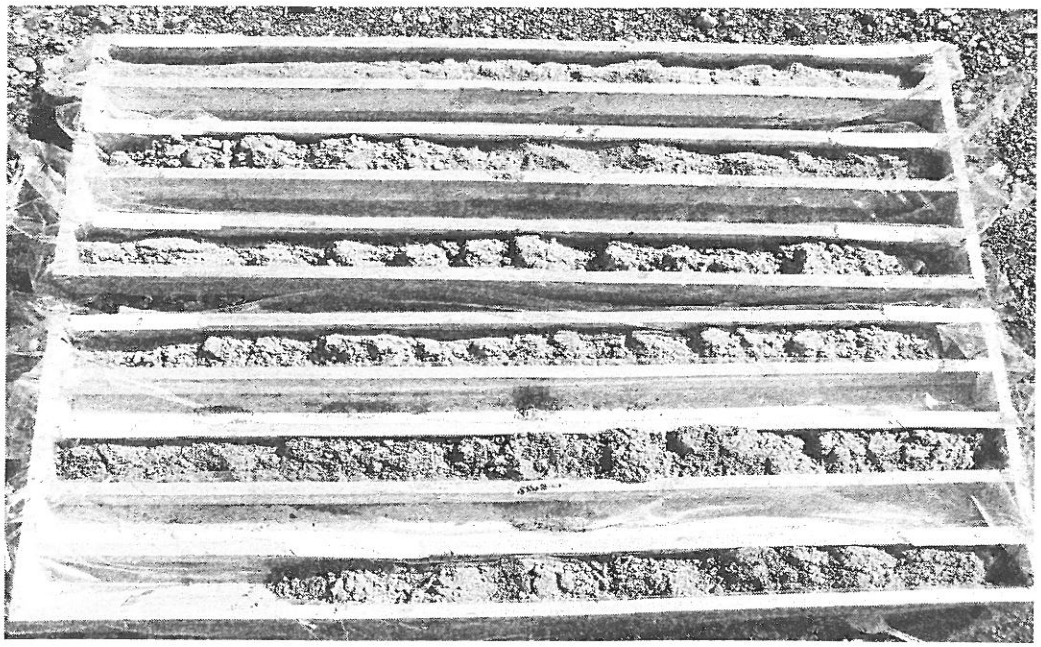


Figure 5. The drilling sediments were removed from drill before collecting to the plastic bags.

Table 1. The sampling depths. The groundwater table was 15.0 m in railway, and 4.6 m in Lahti garden.

Railway		Lahti garden	
Depth (m)	Sediment	Depth (m)	Sediment
0.4	clay	1.2	clay
1.5	clay	2.6	clay
5.7	clay	3.8	clay
10.8	clay	5.7	clay
11.3	sand	14.7	clay
32.4	clay	25.5	sand

2.1.3 Microbial pesticide degradation

Sediments for studies on the microbial atrazine degradation were taken from the depth of 14 m in railway (Lahti). Sediments for studying the microbial BAM degradation were taken from a groundwater well in southern Finland (Fig. 6) using Ekman sampler. In the surrounding area nearby this well, dichlobenil had been used for years before its persistent metabolite BAM was detected in this groundwater well above the drinking water limit of 0.10 µg/l.

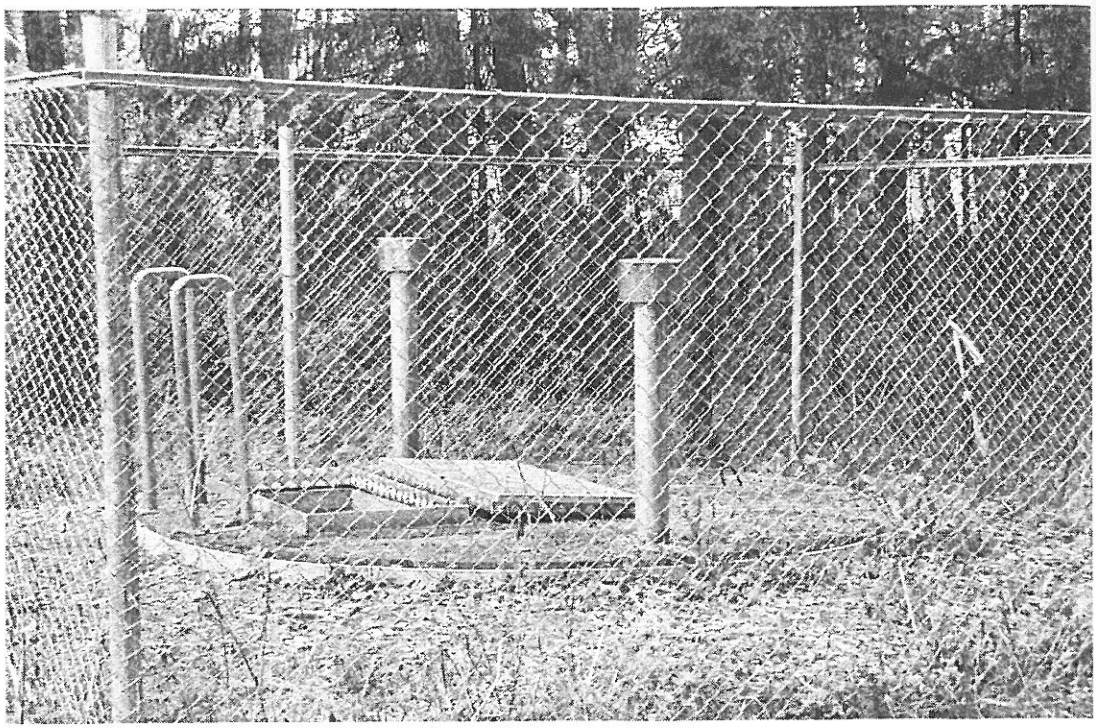


Figure 6. Typical groundwater well in Finland.

2.2 Pesticide analyses

The pesticide extraction from soil was done to (i) confirm the qualitative and quantitative pesticide extraction, (ii) determine where in sediments the pesticides were accumulated, and (iii) determine in degradation experiment whether the pesticides were degraded by microbes or absorbed to the sediments. The pesticides were extracted from 10 g (dw) of soil in triplicates by adding 55 ml of methanol : H_2O (3:1 v/v), and 40 µl of propazine (500 ng/µl) as an internal standard. The sediments were mixed carefully, and tubes were sonicated at 20 °C for 20 minutes (Everest Ultrasonic sonicator, Turkey) to release particles from sediments, followed by shaking overnight (200 rpm) at 21 ± 2 °C. The samples were centrifuged for 10 min at 2500 x (Multifuge 1 S-R Heraeus, Germany),

supernatants were transferred to 50 ml tubes, and evaporated for 18 hours (Christ RVC 2-18 vacuum centrifuge, Germany) to concentrate the pesticides. The extraction steps were repeated for two more times to get the final volume of 2.5 ml of the concentrated samples. 600 μl of samples were filtrated through a 0.45 μm GHP membrane (Acrodisc®, Gelman, Pall Corporation, Ltd., NY, USA) for HPLC analysis, and 20 μl was analyzed by HPLC.

The standards were made from (i) a stock with 30 ng/μl of atrazine, simazine and BAM, (ii) 500 ng/μl propazine (internal standard), and (iii) methanol : H_2O (3:1 v/v) as presented in Table 2. The five standards contained the same amount of propazine as the samples. Based on the internal standard, the concentrations of pesticides in the samples were calculated from the HPLC peak areas.

Table 2. The preparation of standards for the extraction of pesticides from soils. The pesticide stock of 30 ng/μl for each pesticide contained 90 μl of atrazine (500 ng/μl), 90 μl of simazine (500 ng/μl), 90 μl of BAM (500 ng/μl) and 1230 μl of methanol : H_2O (3:1 v/v). The propazine stock concentration was 500 ng/μl, and the ratio of methanol : H_2O was 3:1 (v/v).

Pesticide (ng/μl)	Pesticide stock (μl)	Propazine stock (μl)	methanol : H_2O (μl)
0.5	10	40	550
1	20	40	540
2	40	40	520
5	100	40	460
10	200	40	360
20	400	40	160

In the degradation experiment, the 100 µl of liquid sample was combined with 40 µl of simazine (internal standard, 200 ng/µl) and 460 µl methanol : H_2O (3:1 v/v) for the HPLC analysis. Simazine was used as an internal standard instead of propazine, due to the low simazine ratio of 1/1000 in sediment to that supplemented in standard. The standards were made from (i) a stock with 30 ng/µl of atrazine and BAM, (ii) 200 ng/µl simazine (internal standard), and (iii) methanol : H_2O (3:1 v/v) as presented in Table 3.

Table 3. The preparation of standards for microbial degradation experiments. The pesticide stock of 30 ng/µl for each pesticides contained 90 µl of atrazine (500 ng/µl), 90 µl of BAM (500 ng/µl) + 1320 µl of methanol : H_2O (3:1 v/v). The simazine stock concentration was 200 ng/µl, and the ratio of methanol : H2O was 3:1 (v/v).

Pesticide (ng/µl)	Pesticide stock (µl)	Simazine stock (µl)	methanol : H_2O (µl)
0.5	10	40	550
5	100	40	460
10	200	40	360
15	300	40	260
30	560	40	0

The samples and standards were filtered through a 0.45 µm GHP membrane (Acrodisc®, Gelman, Pall Corporation, Ltd., NY, USA) and 20 µl was analyzed by HPLC equipped with the sample processor (Water 712 WISP, MA, USA), two Chromatography Pumps (model 6000A, Waters Associates Inc, Milford,

Massachusetts, USA), Symmetry C_{18} Column (3.9 * 150 mm, C-18, Waters, MA, USA), and UV detector (Hewlett Packard HP 1050, MI, USA), set at 225 nm for atrazine and simazine analysis, and 215 nm for BAM analysis. The system was controlled with a Maxima 820 chromatography workstation (Millipore, Ventura, California USA), which also performed the data collection from the UV detector. The flow rate of the mobile phase was 1 ml min^{-1} in the whole system. The chromatographic separation was carried out using a linear gradient profile of 10 mM phosphate buffer (pH 7.0), and acetonitrile increasing from 30 % to 70 % in 12 min, hold at 70% for 1 min, and back to 30% in 5 min, and then last for another 5 min at 30%.

2.3 Microbial pesticide degradation in bioreactors

In the biodegradation experiments, soil column with the liquid circulation by pumping (atrazine degradation), and soil suspension in shaker flasks (atrazine and BAM degradation) were used. Each bioreactor consisted of sediment, pesticide (atrazine or BAM, as N sources for microbes), microbes (as potential pesticide degraders), or citrate (as C source for microbes) according to Tables 4 and 5. After the experiments, curves of atrazine and BAM degradation were drawn based on the results from HPLC analysis. The concentration was shown in ng/g dw.

2.3.1 Atrazine degradation in soil columns and soil suspensions

The experimental set-up consisted of 8 treatments in duplicates (soil columns) or triplicates (soil suspensions) (Table 4). The treatment 1 was soil without supplements, and treatment 2 was soil with atrazine addition. In treatments 3, 4, and 5, the bacterial strain added was *Pseudomonas* ADP (DSM 11735), a widely

recognised atrazine degrader (Mandelbaum *et al.*, 1995; Dinamarca *et al.*, 2007). The treatments 6, 7, and 8 were supplemented with the mixture of four microbial strains, which included two bacterial strains, *Pseudomonas* sp. 17.2 and *Pseudomonas* sp. 18.2, and two fungal strains, *Penicillium* sp. 18.2b and *Acremonium* sp. 16.2. These four microbial strains were isolated from sites contaminated by triazine herbicides (Xinxin Liu), and they were potential atrazine degraders. In treatments 3 and 6, only microbes were supplemented, while additional supplements in treatments 4 and 7 were atrazine and in treatments 5 and 8 were atrazine and citrate.

Table 4. Treatments in atrazine degradation experiments. In soil column the final liquid volume was adjusted to 150 ml, and in soil suspension to 50 ml by adding water.

Treatments	1	2	3	4	5	6	7	8
Atrazine (mg/l)	0	100	0	100	100	0	100	100
Microbes (ml)	0	0	1	1	1	4	4	4
Citrate (g/l)	0	0	0	0	1	0	0	1

The soil column system consisted of a 24-channel pump, soil columns, and water supplemented with atrazine in flasks on a shaker (150 rpm) in the room temperature at 21 ± 2 °C (Fig. 7). The pump circulated water at the speed of 1 l/h from flasks to the bottom of soil columns, through the soil, and then back to the flasks. The sediment of 15 g (ww) / 14.7 g (dw) was added into each column. Microbes were inoculated in the middle of the column according to the experimental design presented in Table 4. Atrazine was added to the final

concentration of 100 mg/l from a stock of 10 g/l in methanol : H_2O 3:1, v/v, and sodium citrate of 1 g/l from a 100 g/l stock in deionised water. Sterilized water was added to the flasks to the final volume of 150 ml.

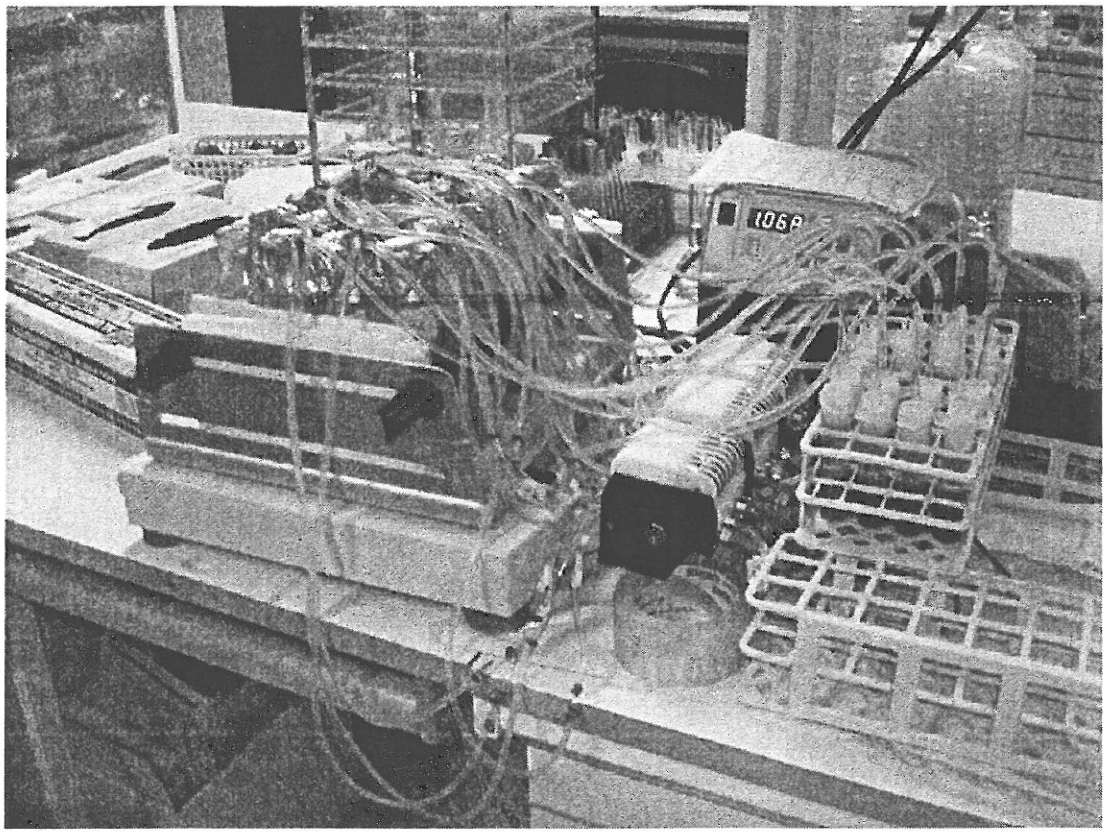


Figure 7. Atrazine degradation in soil columns with circulating water supplemented with atrazine.

In soil suspensions system, 15 g ww (14.7 g dw) of sandy sediment was added into each flask, microbial strains, sodium citrate, and atrazine were supplemented as presented above for soil columns, and water was added to the final volume of 50 ml (Table 4). Flasks were shaken at 200 rpm in the room temperature at 21 ± 2 °C through the experiment. The mass of each flask was weighted both in the beginning and in the end of the experiment to evaluate the evaporation.

The flasks were covered with the aluminium foil. The duration of the experiments was 12 days and samples were taken daily for the HPLC analysis (100 µl) and microbial plating (100 µl).

2.3.2 BAM degradation in soil suspensions

The experiment included nine different treatments with three replicates (Table 5). The treatments were almost the same as for atrazine degradation, with the exception of treatment 3 with BAM and citrate addition. Five supplemented microbial strains were *Zoogloea* sp. K-1A2, *Stenotrophomonas* sp. PK-1B4, *Arthrobacter* sp. KP-1C2, *Cytophaga* sp. PM-1B4, and *Luteifibra* sp. PM-1C2. *Zoogloea* sp. K-1A2 was isolated from the same groundwater well sediment as used in this experiment, and other four strains were isolated from sites contaminated by BAM (Veera Pukkila).

Table 5. Treatments in BAM degradation in soil suspensions. The final liquid volume was adjusted to 50 ml.

Treatments	1	2	3	4	5	6	7	8	9
BAM (mg/l)	0	100	100	0	100	100	0	100	100
Microbes (ml)	0	0	0	1	1	1	4	4	4
Citrate (g/l)	0	0	1	0	0	1	0	0	1

The 82 g ww (14.8 g dw) of sediment was added into each flask, together with 1 g/l of sodium citrate, 100 mg/l of atrazine, and 1 ml of each individual strain, then

sterilized water was added to get the final liquid volume of 50 ml (Table 5). The flasks were incubated in the room temperature at 21 ± 2 °C for 12 days in a shaker at 120 rpm. Samples were taken daily for HPLC analysis (100 µl) and microbial cultivation (100 µl) from one of three parallel flasks.

2.4 Microbial cultivations

2.4.1 Inoculations

The microbial strains known to degrade pesticides as a nitrogen source were cultivated separately for the supplementation in the biodegradation experiments. All equipments and media were autoclave sterilized at 121°C for 20 min. Each strain was cultivated on the selective solid mineral agar (appendixes 1 and 2) with 33 mg/l atrazine or 10 mg/l BAM as the nitrogen source. Plates were incubated for seven days in the room temperature at 21 ± 2 °C, and then each microbial strain was inoculated to two tubes with 10 ml of same liquid medium. The tubes were shaken (200 rpm for atrazine degraders; 120 rpm for BAM degraders) in the room temperature at 21 ± 2 °C for three days. The two tubes of each strain (2 * 10 ml) were inoculated to a 250 ml Erlenmeyer flask containing 100 ml of the same mineral medium, and grown for seven days in the same conditions as the inoculation was cultivated.

In the experimental setup (Tables 4 and 5), either one or four microbial strains were inoculated to the bioreactors and, therefore, four strains were combined during the following washing procedure. The cultures were centrifuged for 10 min at 4000 x g. Supernatants was discarded and 40 ml of 0.9 % NaCl was added. The same washing was repeated for another time. After the centrifugation, 10 ml of

sterile water was added to solubilise one microbial strain, or four microbial strains.

2.4.2 Cultivation of bioreactor microbes

On the first and last days of the experiment, 100 µl of liquid samples were taken from all replicates of treatments without supplementation or with only atrazine or BAM supplementation, and one replicate of the rest treatments for microbial cultivation. During the experiment, samples were taken from one of three parallel experiments daily. The 100 µl samples were diluted from 10^{-1} to 10^{-8} in 0.9 % NaCl (atrazine) or in sterilized water (BAM). Then 100 µl of each dilution was spread on a mineral medium plate, and incubated in room temperature at 21 ± 2 °C until visible growth. The colony forming units (cfu/ml) were calculated according to equation (1), and the plates were stored in refrigerator (4 °C) for identification of cultivated microbes.

$$C = \frac{\sum N_i}{\sum V_i} \qquad (1)$$

C is the weighed average of the microbial density (cfu / ml), N_i is the number of colony forming units on the i:th plate, and V_i is the sample volume on the counted plates.

2.5 Identification of bioreactor microbes

The colonies from the atrazine and BAM degradation experiments were inoculated to the mineral medium agar plates supplemented with pesticides as

nitrogen source, and incubated in the room temperature at 21 ± 2 °C. The colonies with different characteristic visible features (colour, shape) on cultivation plates were re-plated until pure cultures. To identify microbial species degrading atrazine or BAM, the microbial DNA was isolated and 16S rDNA was amplified for sequencing using polymerase chain reaction (PCR).

2.5.1 DNA isolation

The microbes from pure cultures were inoculated on TGY plates containing 5 g/l of tryptone, 1 g/l of glucose, 2.5 g/l of yeast extract, and 15-17 g/l of agar. The plates were incubated for three days in the room temperature at 21 ± 2 °C. Colonies were collected to the Eppendorf tubes and stored in freeze (- 20 °C). The DNA was isolated by following the instructions of Microbial DNA isolation kit (MO BIO Laboratories, Inc. UltraClean^{TM}). In the agarose gel electrophoresis (Appendix 3), the mixture of GeneRuler 1 kb DNA ladder (0.5 mg DNA/ml, 1 µl) and loading buffer (1 µl) was pipetted into the first and last well, and the mixture of each DNA isolation (5 µl) and loading buffer (1 µl) was pipetted into other wells. The electrophoresis was running at 150 V, 400 mA for 30 min, and then a photo was taken under the UV light. DNA was found from all the samples.

2.5.2 Polymerase chain reaction (PCR)

The isolated DNA was used as a template for the polymerase chain reaction (PCR), which consisted of following steps: denaturation, annealing and extension. After the PCR, 5 µl of PCR product was loaded on the agarose gel electrophoresis (Appendix 3) and run at 150 (Appendix 3), 400 mA for 30 min, and then the gel photo was taken under the UV light. The PCR products were sent for sequence analysis.

For the PCR amplification of bacterial 16S rDNA from N different strains, the reagents were prepared as follows, 10x buffer, 5*N µl; dNTP (25 mM), 0.5*N µl; primer pA (10 µM) (AGAGTTTGATCCTGGCTCAG), 0.5*N µl & primer pH (10 µM) (AAGGAGGTGATCCAGCCGCA), 0.5*N µl; DyNAzyme II DNA Polymerase (2.0 U/µl), 1*N µl and sterilised H_2O, 41.5*N µl. The 49 µl portion of the mixture was divided into *N PCR tubes. The template DNA (1 µl) was added to the PCR tubes, expect for the negative control in which autoclaved-sterilized water (1 µl) was used. The final volume in each PCR tube was 50 µl. The PCR program for bacteria was: 94.0 °C, 5 min; and then 25 cycles of 94.0 °C, 20 s; 55.0 °C, 20 s; 72.0 °C, 30 s. Finally, 72 °C, 5 s; 11 °C, 5 s, and then cooled to 4 °C.

For the PCR amplification of ribosomal DNA from N different strains, the reagents were prepared as follows, 10x buffer, 5*N µl; dNTP (25 mM), 1.5*N µl; primer FUN18F (10 µM) (TTGCTCTTCAACGAGGAAT), 0.5*N µl; primer ITS1 (10 µM) (TCCGTAGGTGAACCTGCGG), 0.5*N µl; DyNAzyme II DNA Polymerase (2.0 U/µl), 0.5*N µl and sterilised H_2O, 37*N µl. The 45 µl portion of the mixture was divided into *N PCR tubes. The 5 µl of template DNA was added to the PCR tubes, expect for the negative control where autoclaved-sterilized water (5 µl) was used. The total volume in each PCR tube was 50 µl. The PCR program for fungi was 94.0 °C, 5 min; and then 40 cycles of 94.0 °C, 1 min; 50.0 °C, 30 s; 72.0 °C, 1.5 min. Finally 72 °C was hold for 10 min, followed by 11 °C, 5 min, and then 4 °C.

2.5.3 Sequencing and identification of cultivated microbes

The ribosomal rDNA PCR products were partially sequenced in the Institute of Biotechnology, Viikki (DNA Sequencing Service) using primer pairs of pE (AAACTCAAAGGAATTGACGG) and pF (ACGAGCTGACGACAGCCATG). Sequencing results were processed by Staden Package program and on-line EMBL Fasta program from European Bioinformatics Institute to get the further identification works done.

3. RESULTS

3.1 Pesticides in the subsurface sediments

Extraction efficiencies of atrazine, simazine, and BAM were all above 93 % in pre-experiment. Thereafter all real experimental samples have been extracted in the same way as the pre-experimental samples.

3.1.1 Lahti railway station

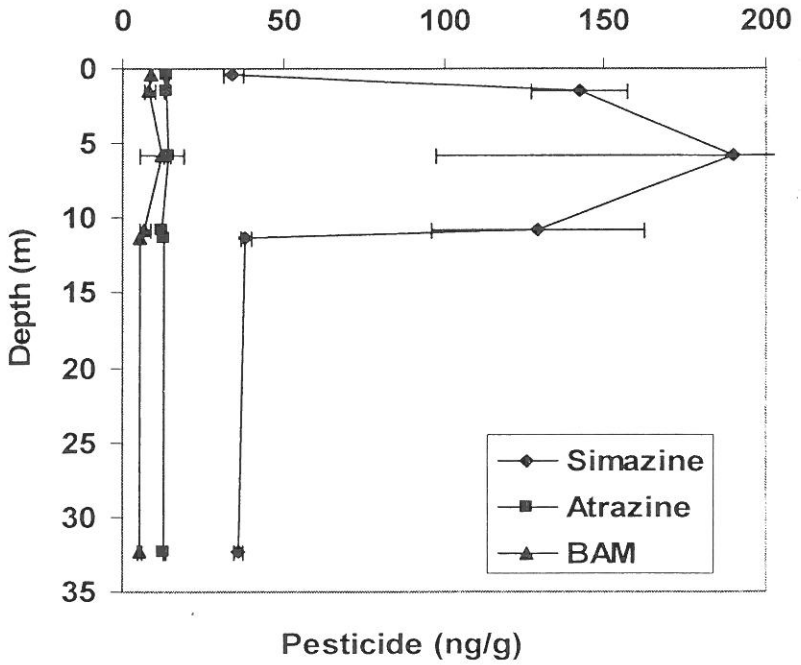


Figure 8. Concentrations of pesticides in subsurface sediments in railway station. The groundwater table was 15 m below the surface.

Pesticides were detected in concentrations of 5.3 ± 0.5 – 189.6 ± 92.1 ng/g dw. Throughout the sampling depths from surface down to 32.4 m, the concentration of atrazine and BAM didn't vary much, and was between 12.2 ± 0.4 – 14.0 ± 1.1 ng/g dw, and 5.3 ± 0.5 – 12.1 ± 6.9 ng/g dw, respectively. Unlikely, the concentration of simazine changed significantly, especially above the groundwater table in 15 m. Above 5.7 m, the concentration of simazine increased remarkably from 34.2 ± 3.1 to 189.6 ± 92.1 ng/g dw, and from 5.7 m down to 11.3 m the concentration then decreased to 38.2 ± 1.8 ng/g dw. Throughout the sampling depths at railway station, simazine always had the highest level among three pesticides, and BAM the lowest, but all of them reached the highest concentrations when the soil type was clay.

3.1.2 Lahti city garden

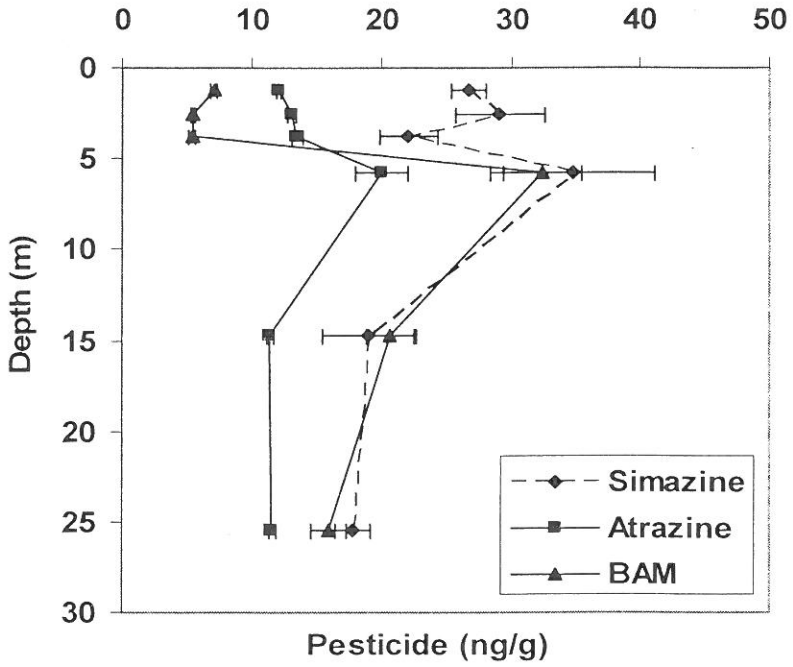


Figure 9. Concentrations of pesticides in subsurface sediments in Lahti city garden. The groundwater table was 4.6 m below the surface.

The concentrations of pesticides varied between 5.34 ± 0.2 – 34.78 ± 6.4 ng/g dw. In the sampling point at Lahti city garden, the changing trends in pesticide concentrations seemed to be quite parallel. At the depth of 5.7 m (around 1 m below the groundwater table at sampling time) where the clay sediment was present, all studied pesticides reached the highest concentrations, which were 34.8 ± 6.4 ng/g dw for simazine, 19.9 ± 2.0 ng/g dw for atrazine, and 32.4 ± 3.1 ng/g dw for BAM. From the depths of 14.7 m to 25.5 m, all pesticides spread quite evenly, simazine concentration was 18.9 ± 3.5 to 17.7 ± 1.3 ng/g dw, atrazine concentration was more or less the same between 11.4 ± 0.3 and 11.5 ± 0.2 ng/g

dw, while BAM concentration decreased from 20.7 ± 2.0 to 15.9 ± 1.4 ng/g dw. Unexpectedly, BAM was found to have a relatively high level in Lahti city garden, and its concentration varied quite sharply between the depths of 2.6 m (5.34 ± 0.2 ng/g dw) to 5.7 m (32.4 ± 3.1 ng/g dw) compared with atrazine and simazine.

3.2 Pesticide degradation in soils

3.2.1 Atrazine degradation in soil columns

A rapid degradation of atrazine was found in all soil column treatments. In treatment 2 (Fig. 10A) with only atrazine supplementation, 41.0 ± 3.0 % of atrazine disappeared within the first day, and the degradation continued from the first day to the second day with a further portion of 9.68 ± 4.8 % atrazine degradation. After that the concentration of atrazine was almost the same throughout the whole experiment, which was 11.2 ± 3.2 % in average. This indicated that there might be microbes in sediments which can use atrazine as a nitrogen source.

As seen in Fig. 10B, in treatments 4 and 5 with atrazine and atrazine degrading *Pseudomonas* ADP, 60.6 ± 2.2 % and 82.1 ± 3.4 % of the supplemented atrazine was degraded within one day, after that atrazine concentration was kept quite stable from days 1 to 11, the average amounts were 11.0 ± 2.6 %, and 11.1 ± 3.4 %, respectively. Compared with treatment 4 (atrazine and *Pseudomonas* ADP), the additional carbon source in treatment 5, sodium citrate, did not affect the degradation.

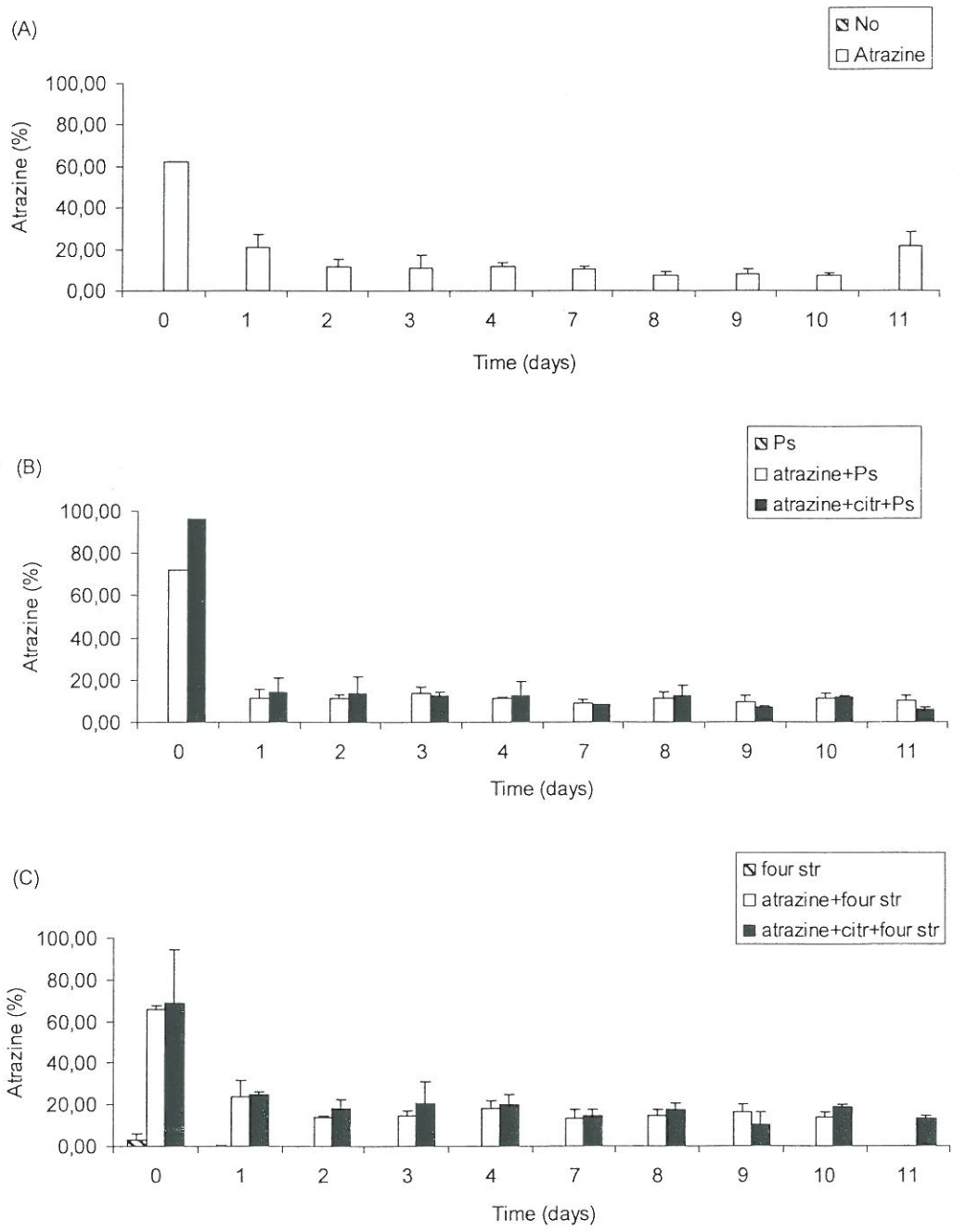


Figure 10. Atrazine degradation in soil column. (A) Treatment 1: no supplementation; Treatment 2: atrazine, (B) Treatment 3: *Pseudomonas* ADP; Treatment 4: atrazine and *Pseudomonas* ADP; Treatment 5: atrazine, *Pseudomonas* ADP, and citrate, (C) Treatment 6: four strains; Treatment 7: atrazine and four strains; Treatment 8: atrazine, four strains, and citrate.

In treatments 7 and 8 (Fig. 10C), four microbial strains were added. The supplemented atrazine was degraded rapidly within the first day (about 40 %), but not as fast as in the treatments 4 and 5 in which *Pseudomonas* ADP (DSM 11735) only was added. From days 1 to 11, the average concentrations of atrazine stayed at 14.4 ± 3.1 % in treatment 7 supplemented with atrazine and four microbial strains, and 17.7 ± 3.8 % in treatment 8 supplemented with atrazine, citrate, and four microbial strains. Similarly, no obvious degradation was found since the second day until the end of the experiment.

3.2.2 Atrazine degradation in soil suspensions

The atrazine degradation in soils suspensions was less than in soil columns with the water circulation. When only atrazine was added (Fig. 11A), 27.9 ± 10.4 % of supplemented atrazine was degraded within the first day. Then, between days 1 to 11, atrazine concentration was kept stable around 51.3 ± 9.4 %. The results supported that the sediment contained microbes capable of degrading atrazine, which was also found in the soil column experiments.

In the rest of soil suspension treatments (Fig. 11B & 11C), the degradation happened rapidly during the first day of the experiment. 22.6 ± 8.4 %, 30.7 ± 11.8 %, 16.0 ± 6.7 %, and 19.2 ± 12.9 % of atrazine was degraded in treatments 4 (atrazine and *Pseudomonas*), 5 (atrazine, citrate and *Pseudomonas*), 7 (atrazine and four strains), and 8 (atrazine, four strains, and citrate), respectively. Again, sodium citrate was found to have no significant impact on the atrazine degradation.

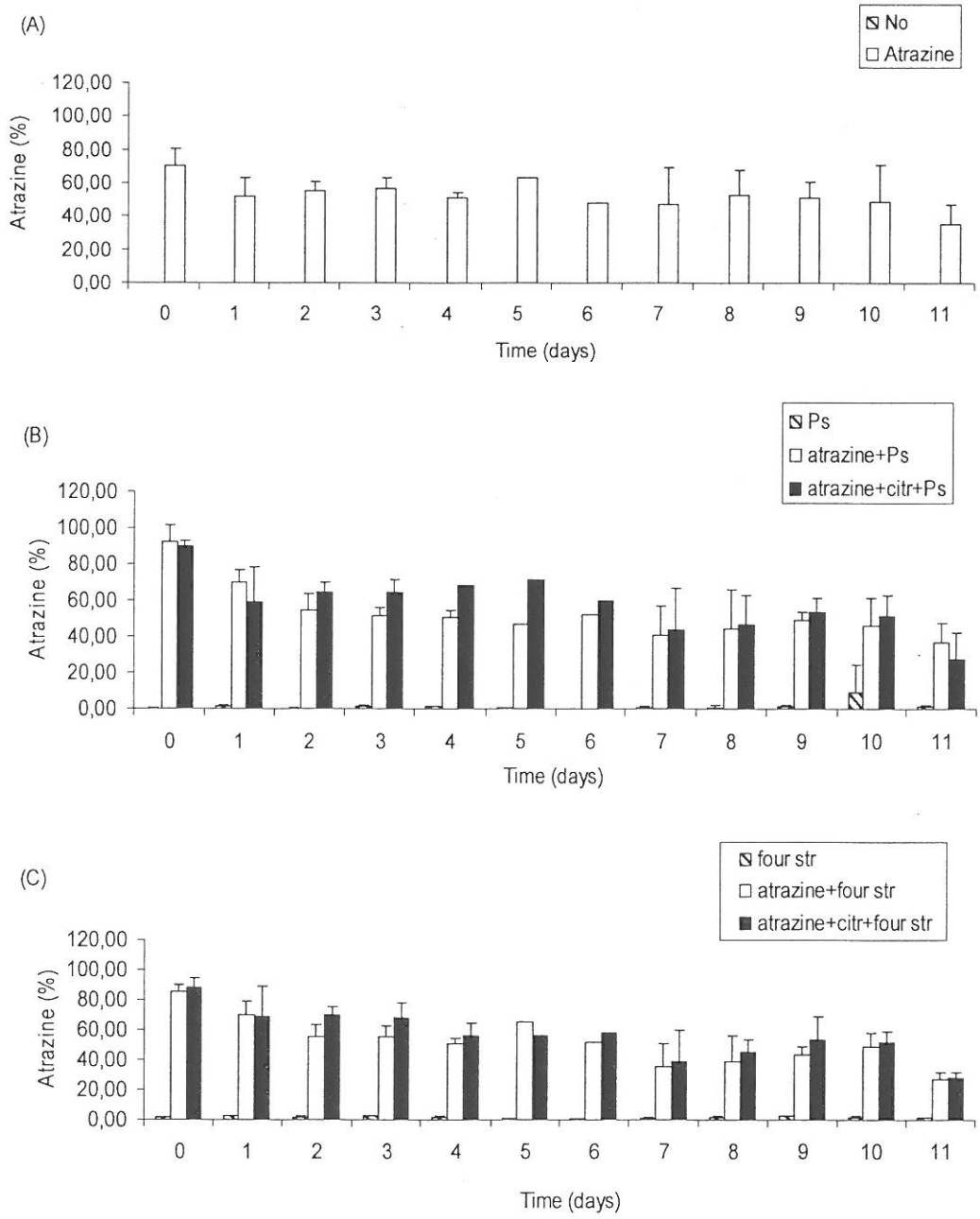


Figure 11. Atrazine degradation in soil suspension. (A) Treatment 1: no supplementation; Treatment 2: atrazine, (B) Treatment 3: *Pseudomonas* ADP; Treatment 4: atrazine and *Pseudomonas* ADP; Treatment 5: atrazine, *Pseudomonas* ADP, and citrate, (C) Treatment 6: four strains; Treatment 7: atrazine and four strains; Treatment 8: atrazine, four strains, and citrate.

3.2.3 BAM degradation in soil suspensions

Within the first day, in treatment 2 with BAM supplementation 36.1 ± 9.2 % of BAM was degraded; and in treatment 3 with citrate and BAM supplementations 33.2 ± 5.4 % of the supplemented BAM was degraded (Fig. 12A). The most significant BAM degradation within the first day happened in treatments 5 and 6 where *Zoogloea* sp. K-1A2 was added. As shown in Fig. 12B, after the first day, 50.7 ± 5.1 % of the supplemented BAM was degraded by the contribution of *Zoogloea* sp. K-1A2, and the microbes originally from the sediment, while 57.5 ± 8.4 % of BAM was degraded under the function of *Zoogloea* sp. K-1A2, citrate, and microbes in sediments. *Zoogloea* sp. K-1A2 seemed to have more effects on the BAM degradation than the other four potential degraders, because only 35.8 ± 9.0 % and 28.7 ± 2.6 % of the supplemented BAM was degraded within the first day in treatments 8 and 9 (Fig. 12C) where the four microbial strains were added.

In all treatments, BAM was degraded fast within the first day. BAM concentrations were almost the same between days 1 to 10, which were 49.5 ± 4.5 % in treatment with only BAM supplementation, 52.1 ± 3.8 % in treatment with BAM and citrate supplementation, 51.3 ± 4.3 % in treatment in which BAM and *Zoogloea* sp. K-1A2 were added, and 51.3 ± 4.5 % in treatment with supplemented BAM, citrate, and *Zoogloea* sp. K-1A2. Similarly, 51.1 ± 4.4 % of BAM was kept in treatment with additional BAM and four microbial strains, and 50.1 ± 6.3 % in treatment with the supplementations of BAM, citrate, and four microbial strains from day 1 to day 10. Added sodium citrate did not have any obvious positive influence on the degradation.

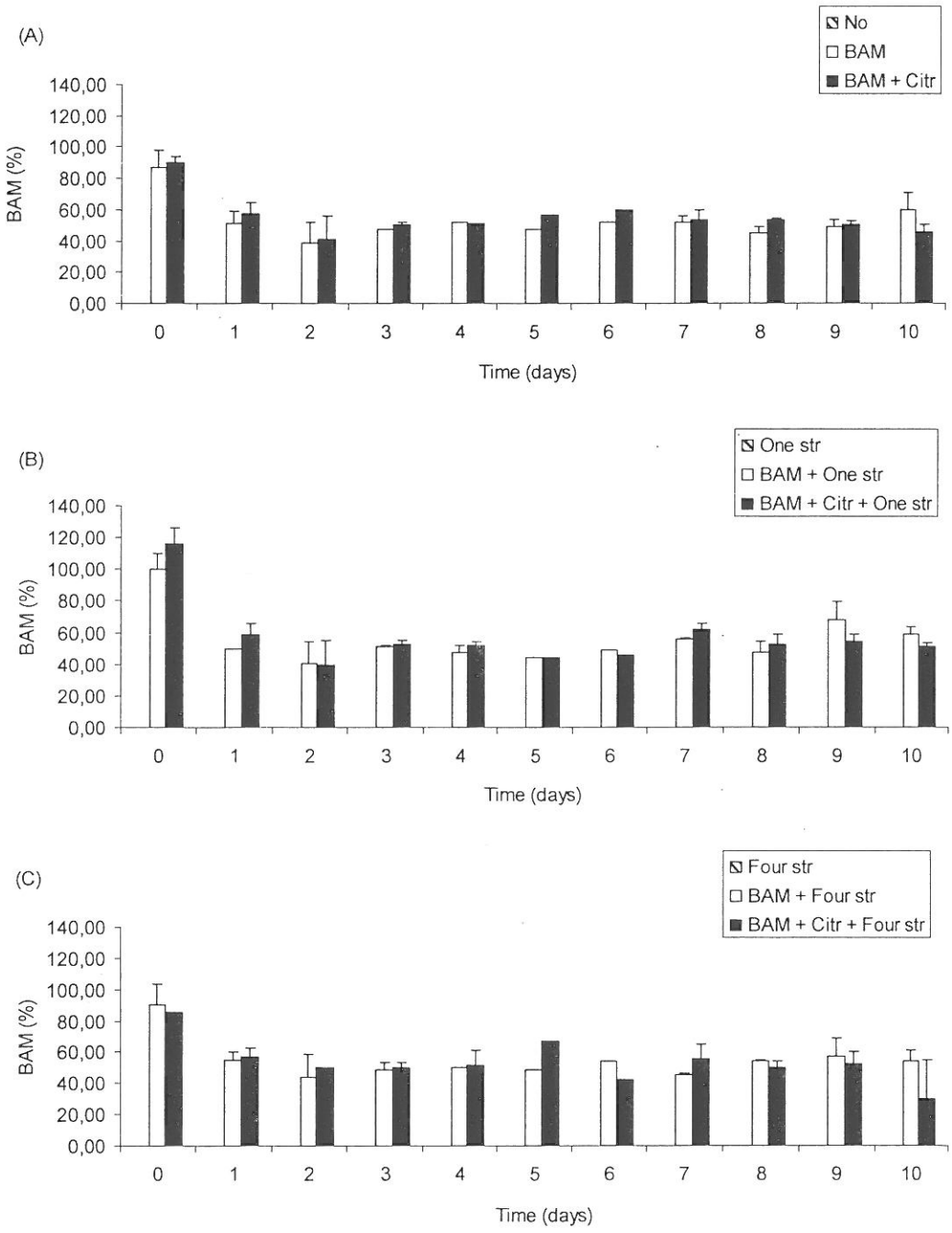


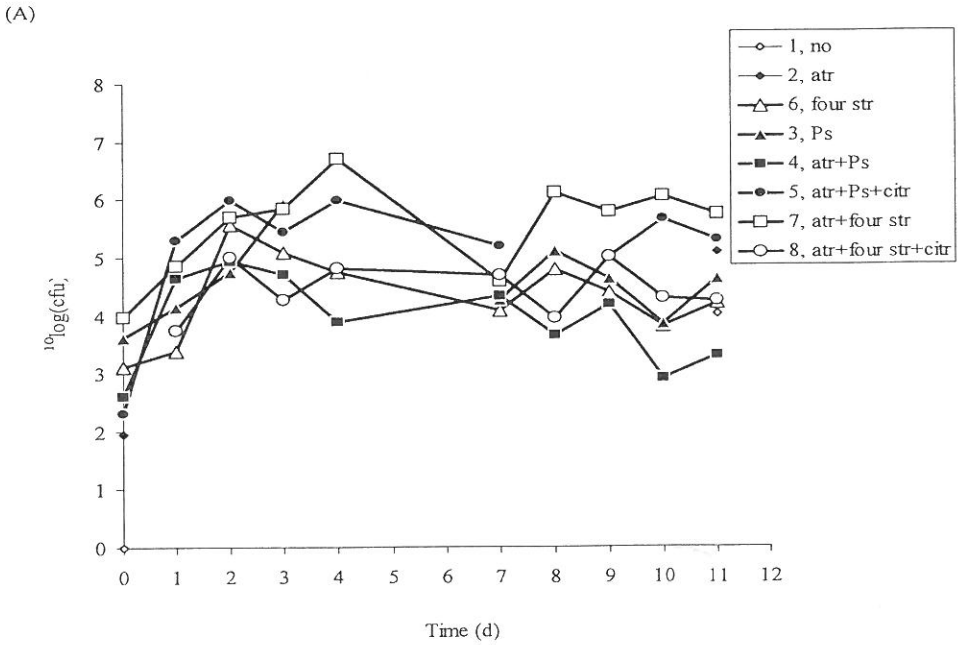
Figure 12. BAM degradation in soil suspension. (A) Treatment 1: no supplementation; Treatment 2: BAM; Treatment 3: BAM and citrate, (B) Treatment 4: *Zoogloea* sp.; Treatment 5: BAM and Zoogloea sp.; Treatment 6: BAM, *Zoogloea* sp., and citrate, (C) Treatment 7: four strains; Treatment 8: BAM and four strains; Treatment 9: BAM, four strains, and citrate.

3.2.4 Atrazine and BAM extraction from bioreactor sediments

During the experiment, sodium citrate coloured the liquid in bioreactors from clear to brown in all treatments. Based on the HPLC results, no atrazine or BAM was found in sediments in the end of experiment. The pesticides were degraded by microbes, but not absorbed to the sediments in all treatments.

3.3 Pesticide degrading microbes

3.3.1 Atrazine degrading microbes



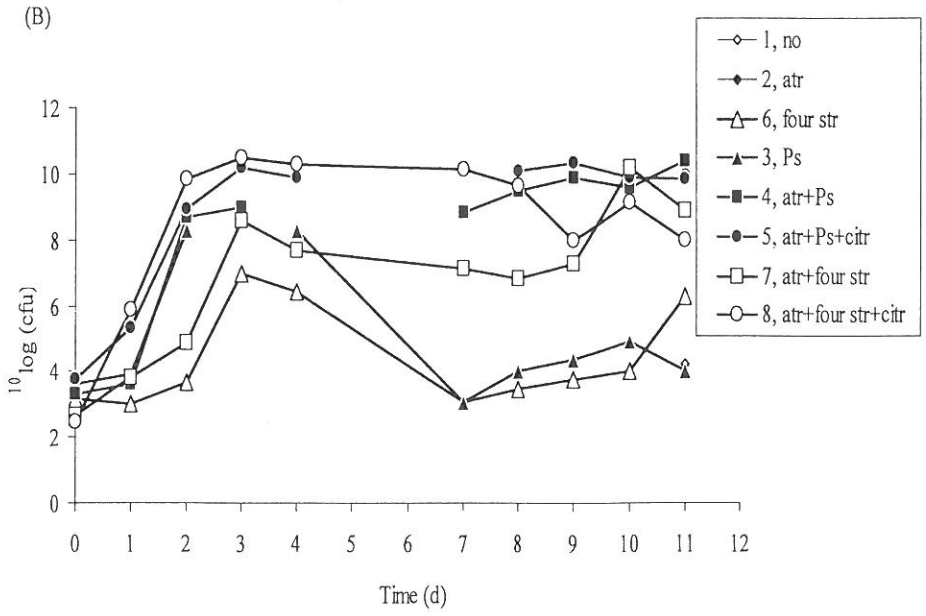


Figure 13. Microbes growing on atrazine as a nitrogen source in soil columns (A) and soil suspensions (B). Digit indicated the treatments.

Microbial growth

In the soil column experiment (Fig. 13A), it can be seen that microbes in treatments with supplemented atrazine grew better than in treatments without supplementation. The microbes from sediment could also use atrazine as a nitrogen source to stimulate their growth. Sodium citrate seemed to have clear positive influence on the growth of *Pseudomonas* ADP, but not when four microbial strains were supplemented. But *Pseudomonas* ADP were not capable to use atrazine as well as microbial mixtures because in the end of the experiment, the microbial number in treatment with supplemented atrazine and microbial mixture was 33 times more than in treatment only with the microbial mixtures. *Pseudomonas* ADP grew better without the addition of atrazine as a nitrogen source.

In soils suspensions, atrazine was clearly used by soil microbes as the nitrogen source. Sodium citrate had neither clear effect on the growth of *Pseudomonas* ADP (Fig. 13B, treatments 4 & 5), nor on the growth of microbial mixtures (Fig. 13B, treatments 7 & 8). In the end of experiment, in treatment 4 where atrazine and *Pseudomonas* ADP were added, the microbial quantity was 2.38×10^{10} cfu/ml. In treatment 3 with only *Pseudomonas* ADP supplementation, the microbial quantity was 1.09×10^{4} cfu/ml. Atrazine also stimulated the growth of four strains significantly, which can be concluded from the comparison between treatments 6 (four microbial strains) and 7 (atrazine and four microbial strains).

Identification of microbes

In the end of bioreactor experiments, five colonies with different outlooks were isolated and purified to characterize the strains. The 16S rDNA was amplified by PCR as seen in Figure 14 from wells 2 to 6, and sequenced.

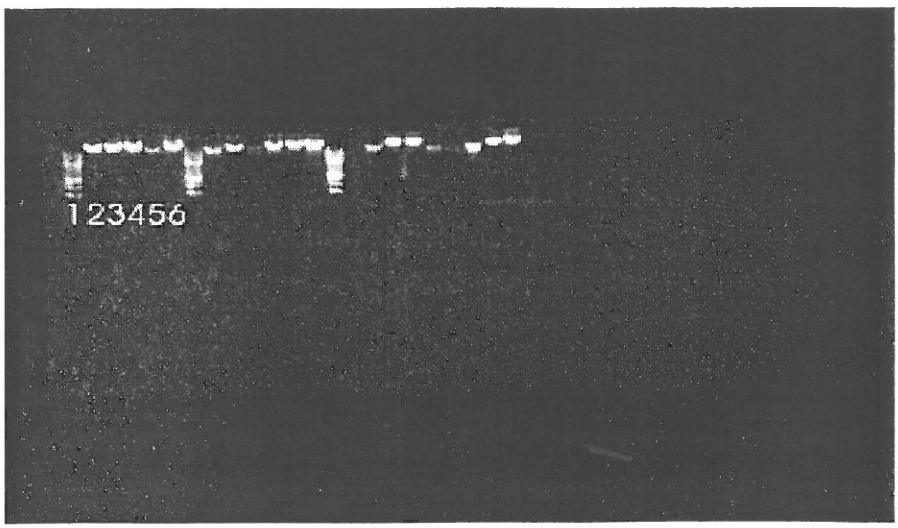


Figure 14. 16S rDNA PCR products of selective microbial strains growing on atrazine as a nitrogen source.

Table 6. Identification of strains
from atrazine degradation experiment.

Name	Identity - %
Rhodococcus fascians	99.878
Pseudomonas sp.	100
Methylobacterium sp.	99.891
Pseudomonas sp.	100
Pseudomonas sp.	99.877

The comparison with the database showed that the isolated atrazine degrading strains were identified as *Rhodococcus fascians*, three *Pseudomonas* sp., and *Methylobacterium* sp. (Table 6). *R. fascians* and *Methylobacterium* sp. were different from those added into the bioreactors in the beginning of the experiment.

3.3.2 BAM degrading microbes

Microbial growth

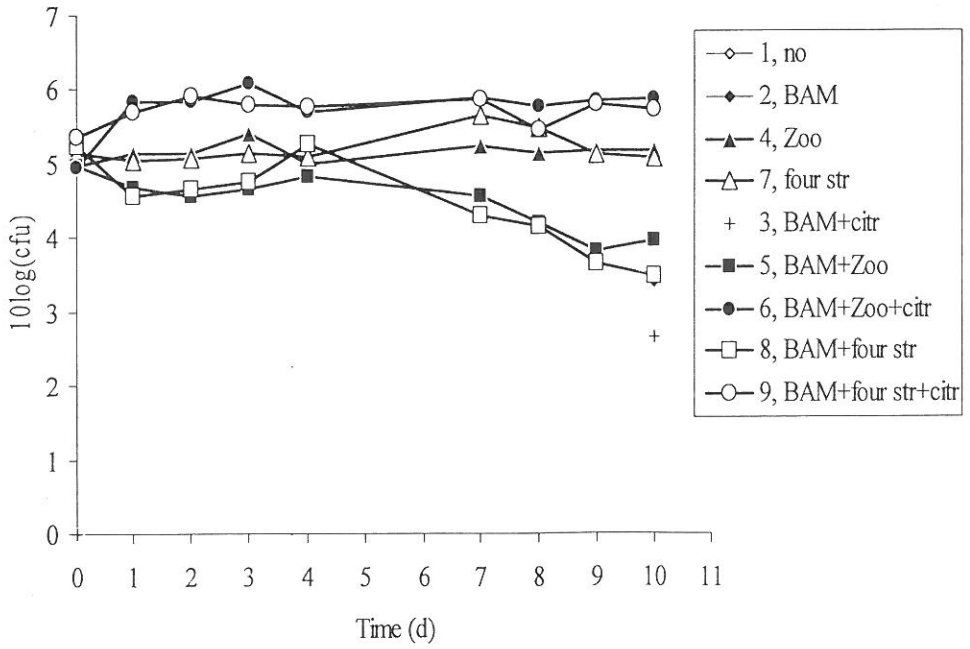


Figure 15. Microbes growing on BAM as a nitrogen source in soil suspensions.

In the BAM degradation experiment, in treatment 2 with BAM supplementation, the microbial quantity was lower than in treatment 1 without any addition. Based on the additional sodium citrate, in the end of the experiment, 81 times more microbes was found in treatment 6 (BAM + *Zoogloea* sp. + citrate), compared with in treatment 5 (BAM + *Zoogloea* sp.). Similar results based on the additional citrate were found in the treatments with four strains. Citrate had a positive influence on the microbial growth under the BAM supplementation, no matter

there was one strain or four strains. Unlike the effects of sodium citrate, the additional BAM seemed to act negatively by itself to the microbial growth. On the last day of experiment, there was around 16 and 39 times less microbes in treatments 5 (BAM + *Zoogloea* sp.) and 8 (BAM and four microbial strains) than in treatment 4 (*Zoogloea* sp.) and 7 (four microbial strains), respectively.

Identification of microbes

In the end of bioreactor experiments, colonies with different outlooks were isolated and purified to be characterized. The 16S rDNA was amplified (Fig. 16), and sequenced. All bacterial strains were different from those added to the bioreactors. They were three *Pseudomonas* strains, *Pseudomonas putida* strain PC, *Pseudomonas mandelii*, and *Pseudomonas* sp. PH-03, and one *Sphingomonas* strain, *Sphingomonas* sp. TSBY-34 (Table 7).

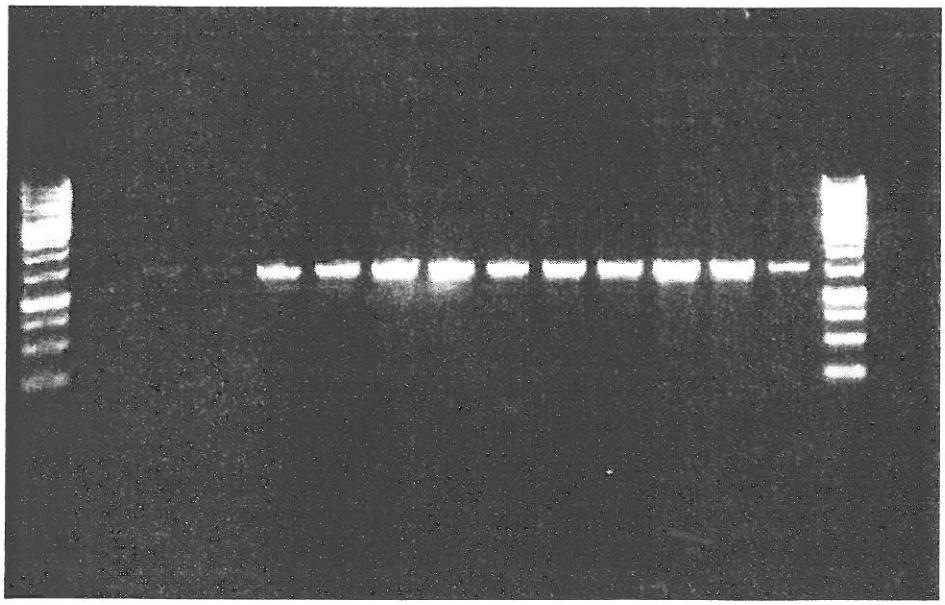


Figure 16. 16S rDNA PCR products of selective microbial strains growing on BAM as a nitrogen source.

Table 7. Identification of strains
from BAM degradation experiment

Name	Identity - %
Pseudomonas putida strain PC	100
Pseudomonas mandelii	100
Sphingomonas sp. TSBY-34	100
Pseudomonas sp. PH-03	100

4. DISCUSSION

4.1 Pesticides in the subsurface sediments

The groundwater table at railway station was 15 m at the sampling time. Between surface and the depth of 10.8 m, clay was the only sediment type. The clay between these depths contained the highest amounts of all three target pesticides, simazine, atrazine, and BAM. The sediment type changed to sand at 11.3 m below the surface, and, at this depth, the pesticide concentrations decreased greatly. Especially simazine concentration decreased from 129.6 ± 33.4 to 38.2 ± 1.8 ng/g dw within less than 1 m depth difference. The pesticides are much more easily bound to the sediment type such as clay. Below the depth of 11.3 m down to the bottom of the sampling depth, the concentrations of all pesticides seemed to be quite stable. A small portion of pesticides passed through the sand to the

groundwater, and spread quite evenly throughout the rest of the depths. A large portion of pesticides stayed above the groundwater table.

In Lahti city garden, the highest concentration of BAM, 32.4 ± 3.1 ng/g dw, was almost three times greater than the highest concentration in railway station. The Laune groundwater flows through Lahti City Garden, and at the sampling time, the groundwater table was 4.6 m at the sampling place at city garden. All pesticide concentrations reached the highest levels at the depth of 5.7 m, which was about 1 m below the groundwater table. After leaching into the groundwater, some portions of pesticides passed into the deep sediment, meanwhile some portions of pesticides spread by the groundwater flow. BAM has been found in some Finnish groundwater wells with the groundwater flow.

We need to keep in mind that the groundwater table varies over the years. It can be affected by many aspects such as the rain fall, the soil and rock layers. The groundwater table influences the rate of elution of pesticides into the groundwater.

4.2 Pesticides degradation

Generally, the atrazine concentration dropped rapidly within one day both in soil column and soil suspension experiments. The fastest degradation during the first day was found in the soil column experimental treatments 4 and 5 using atrazine and *Pseudomonas* ADP supplementations, with citrate addition (60.6 ± 2.2 % degraded), or without citrate addition (82.1 ± 3.4 % degraded). Thereafter, the atrazine concentration remained at about the same level until the end of the experiment in all treatments. Mandelbaum *et al.*, (1995) also reported that the concentration of atrazine dropped to close to 30 % within 25 hours from the

beginning of the experiment. There could be two reasons for the rapid disappearance of atrazine. Atrazine was first degraded quickly by microbes, and then absorbed to the sediment. However, no atrazine was found even in the control treatments when the concentration of atrazine in the sediment was determined in the end of experiment. In this case, atrazine was likely degraded by microbes.

Similar to the atrazine degradation, BAM concentration also decreased during the first two days without absorption to the sediment, according to the pesticide analysis in the end of the experiment. The result was surprising, due to some other studies where BAM has been very stable and difficult to degrade, for example Holtze *et al.,* (2006) have concluded in their study that potential for dichlobenil degradation to BAM was found commonly in soil bacteria, whereas further degradation of the BAM intermediate could not be demonstrated. Altogether, atrazine and BAM were clearly degraded in our experiment, by both microbes from the sediments, and the supplemented microbes.

Carbon source

Citrate was added as a carbon source for microbes. Generally, microbes in treatments with supplemented citrate grew better than those without citrate. The carbons in citrate might be more easily to be used by microbes compared with other carbons sources in sediments, for example in atrazine or BAM. Although sodium citrate increased the microbial numbers, the degradation of atrazine and BAM was not affected. In some other studies, sodium citrate has stimulated the atrazine metabolization by *Pseudomonas* ADP, and the size of microbial population has been increased (Mandelbaum *et al.,* 1993a; 1995, Ralebitso *et al.,* 2002). One explanation can be that the duration of our experiment was not enough

for the microbes to adapt to the new environment, and further use the additional carbon source to degrade atrazine and BAM. Another problem was that the addition of sodium citrate into bioreactors caused the coloured liquid, which is not a desired phenomenon in the bioremediation of groundwater.

Microbes

From the treatments without additional microbes, it could be observed that the sediments likely contained microbes able to use atrazine or BAM as the nitrogen source. Indeed, the bacterial strain *R. fascians*, a known atrazine degrader was isolated from the control treatments without any microbial supplementation. Interests were put on studying whether the additional bacterial or fungal strains helped in the pesticide degradation. In case of atrazine, generally, more atrazine was degraded in the treatments with the single microbial addition of *Pseudomonas* ADP (DSM 11735), a published atrazine degrader, compared to the treatments with the addition of four microbial strains. This conclusion was partly agreed with Mandelbaum *et al.*, (1995) who used *Pseudomonas* ADP in the study of degradation of atrazine, the concentration of atrazine dropped to 30 % until the end of experiment.

To study whether the additional microbial mixture succeeded to compete with the soil microbes, the species composition was determined in the end of experiment. Expect for *Pseudomonas* sp., all isolated atrazine degraders were not among the microbial strains supplemented into the bioreactors. Apparently the strains are atrazine degraders originally present in the contaminated site. But as we know, contamination may cause selection pressure in the microbial community and accelerate adaptation. The microbes have shorter reproduction cycles and are more prone to mutations and genetic adaptation than many other organisms. The

microbial adaptation may have occurred in the atrazine contaminated sediment naturally, or even during this experiment, which brought us to the mystery where the microbial degraders actually are from.

During the experiment, the coexistence of bacteria and fungi was found on the basic of growth on the agar medium. The bacteria had grown inside of the fungus hyphae. This indicated that instead of using atrazine, some of the fungi may have used bacteria as a nitrogen source. On the other hand, the bacteria which were used by fungi might be among of those ones which are capable of using atrazine as a nitrogen source. This could be one of the reasons to explain why better degradation was found in treatments with single supplemented strain, but not treatments with the mixture of bacterial and fungus strains.

Since there was no published BAM degraders, *Zoogloea* sp. isolated from the contaminated sediment was added as the single strain into some treatments. Degradation of BAM was found also in the treatments without microbial addition, 36.1 ± 9.2 % of added BAM was degraded within one day. Similarly, significant degradation happened in the treatments with only *Zoogloea* sp. addition, 50.7 ± 5.1 % of the supplemented BAM was degraded within the first day, and 57.5 ± 8.4 % of added BAM was degraded in treatment with *Zoogloea* sp. K-1A2 and citrate supplementations. Based on the sequencing results, all BAM degraders found in the end of experiment were different from those added into the bioreactors. The hypothesis can be again made that the soil microbes were better in competition compared with the supplemented strains.

Soil column

Apparently much more atrazine was degraded in soil columns with circulating liquid inside than in soil suspensions. The reason could be that the water circulation is more close to the natural environment. The nature is much more complicated than the 'micro' environment in the flasks. Compared with in the soil columns with circulating liquid, microbes in the flasks need longer time and more space to get adapted to the new environment in order to play their roles. The soil column offered this opportunity to microbes which in some case increased the atrazine degradation rate.

4.3 Possibility of application into field

After this study, facing the challenge of how to apply the working system into the environment, many factors need to be taken account, such as the citrate, and microbes listed above. The nature is in such a balance that any aliens may disturb the balance and then bring us serious problems. For instance, if the additional carbon and nitrogen sources stimulate the growth of soil and alien microbes, the degradation rate of the contaminants such as pesticides becomes faster also. Thereafter, the toxicity problems of the potential microbial degraders' supernatants (i.e. *Pseudomonas putida* strain), might be more serious to the soil microbes, nematode, and protozoa. Although there are many difficulties when we think about the possibilities of the application into field, processes from some other associated studies are always offering some hints an ideas for us. Study on applying electricity on *in-situ* bioremediation is a brand new topic. For example if we think about applying water circulation system to speed up the degradation of

pesticides and some other contaminants in the places where the groundwater flow does not go, anodes and cathodes can actually be installed in the two edges of the interested area to stimulate the electronic flow together with the water flow, to reach the aim of higher the degradation rate of contaminants. Meanwhile, some monitor can be installed *in-situ* to get the real-time situation.

5. CONCLUSIONS

The results of this study indicate that the degradation of atrazine and BAM did happen in subsurface sediments, and the microbial analyses brought us a few new strains which were not recognized as the degraders before. It would be quite interesting to continue the future study by adding those microbial degraders into different treatments to detect the efficiency of the degradation. The bacterial strain *R. fascians* found in atrazine degradation experiment can be another studying attraction. It can be tested whether it can use bacteria as a nitrogen source, and how quickly it can degrade atrazine. After this experiment, from many ways we should think about how to make the study better. We should find out some other chemicals as the suitable carbon source, the experiment should be taken for a longer time to figure out if the degradation can continue, and more thoughts should be put on how to apply this technique from laboratory to the open field.

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Appendix 1. Mineral medium used in atrazine degradation experiment.

	Compound	MW	concentration			weight g/L		volume	volume to medium 1L
stock1 1000x	CoSO4 x 7 H2O	281.1	2.0	mg / L		2	g	250ml (filter sterilise)	1ml / L
	ZnSO4 x 7 H2O	287.5	2.0	mg / L		2	g		
	CuSO4	159.6	0.2	mg / L		0.2	g		
	Na2WO4	293.8	0.2	mg / L		0.2	g		
	Na2SeO3	172.9	0.2	mg / L		0.2	g		
	NaMoO4 x 2H2O	219.0	0.1	mg / L		0.2	g		
	NiCl2 x 6 H2O	237.7	0.2	mg / L		0.2	g		
	H3BO3	61.8	0.05	mg / L		0.05	g		
	NaVO3	139.9	0.1	mg / L		0.1	g		
stock2 100x	MgSO4	120.4	12.0	mg / L		0.12	g	1L	10ml / L
	MnSO4 x H2O	184.0	10.0	mg / L		0.1	g		
	CaSO4	136.1	9.0	mg / L		0.09	g		
	FeSO4 x 7H2O	278.0	5.5	mg / L		0.055	g		
stock3 1x	K2HPO4	174.2	1.6	g / L		1.6	g	4 L	1000ml / L
	NaH2PO4	120.0	400	mg / L		0.4	g		
stock4 100x	sodium citrate	294.1	1.0	g / L		10.0	g	600ml	10ml / L
stock5	Atrazine	215.7	100	mg / L					3.3ml / L

agar 15 g/L in stock 3

Appendix 2. Mineral medium used in BAM degradation experiment.

	Compound	MW	concentration		weight g/L		volume	volume to medium 1L
stock1 500x	CoSO4 x 7 H2O	281,1	2,0	mg / L	2	g	200ml	2ml / L
	ZnSO4 x 7 H2O	287,5	2,0	mg / L	2	g		
	CuSO4	159,6	0,2	mg / L	0,2	g		
	Na2WO4	293,8	0,2	mg / L	0,2	g		
	Na2SeO3	172,9	0,2	mg / L	0,2	g		
	NaMoO4 x 2H2O	219,0	0,1	mg / L	0,1	g		
	NiCl2 x 6 H2O	237,7	0,2	mg / L	0,2	g		
	H3BO3	61,8	0,05	mg / L	0,05	g		
	NaVO3	139,9	0,1	mg / L	0,1	g		
stock2 100x	MgSO4	120,4	12,0	mg / L	2,464	g	100 ml	10ml / L
	MnSO4 x H2O	184,0	10,0	mg / L	1	g		
	CaSO4	136,1	9,0	mg / L	1,14	g		
stock3 100x	FeSO4 x 7H2O	278,0	5,5	mg / L	0,55	g	100 ml	10 ml/L
stock4 1x	K2HPO4	174,2	1,6	g / L	1,6	g	0,75 L	1000ml / L
	NaH2PO4 x H2O	138,0	400,0	mg / L	0,46	g		
stock5 100x	sodium citrate	294,1	1,0	g / L	100,0	g	600ml	10ml / L
stock6	BAM		100,0	mg / L				10 ml / L
stock7 1000x	thiamine	337,3	1,0	g / L	1	g	50 ml	1ml / L
	biotin	244,3	0,4	mg / L	0,4	g		

agar 15g / L in stock 4

Appendix 3. Instructions for 1 % agarose gel

2 g	agarose
200 ml	1 x TAE buffer

TAE buffer is a buffer solution used in agarose electrophoresis, typically for the separation of nucleic acids such as DNA and RNA. It is made up of Tris-acetate buffer, usually at pH 8.0, and EDTA, which sequesters divalent cations.

Microwave until it has boiled about five times. Cool down to about 50 °C. Add 20 µl ethidium bromide. Tape the gel plate and pour the gel. Wait one hour to solidify.

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No. 20 2004 HÖLSÄ JENNI: Kompostin sieniyhteisön kehittyminen ja karakterisointi molekyylibiologisin menetelmin (pro gradu)

No. 21 2004 MÄKINEN JUSSI: Vesijärven kilpiäistenpohjan ekologinen tila ja hoitosuunnitelma (pro gradu)

No. 22 2004 LÖNNGREN HANNA: Ympäristöasioiden hallinta kaupunkikuntien toimintayksiköissä – Tapaustutkimuksena Hyvinkään kaupungin tilapalvelu (pro gradu)

No. 23 2004 HUOTARI JUSSI : *DAPHNIA LONGISPINA* ja *D. PULEX*- vesikirppujen lisääntyminen simuloiduissa ilmastonmuutos olosuhteissa: ekosysteemitason co_2-altistus (pro gradu)

No. 24 2004 SAARINEN TUOMAS: Petoäyriäisen merkitys Vesijärven ulappavyöhykkeen eläinplanktonyhteisössä (pro gradu)

No. 25 2004 VAKKILAINEN KIRSI: The role of zooplankton in controlling phytoplankton biomass in lake littoral (lis.tutkimus)

No. 26 2004 HAGNER MARLEENA: Koivutisle torjunta-aineena: vaikutukset lehtokotiloon (Arianta arbustorum) ja maaperään (pro gradu)

No. 27 2004 JÄRVINEN KIRSI: Vesikasvien pintojen ja pehmeiden pohjien päällys- ja pohjaeläimistöt matalien järvien tilan kuvaajina (pro gradu)

No. 28 2004 SUUTARI MILLA: Kasviplanktonin ravinnerajoitteisuus Lahden Vesijärvessä kesällä 2000 (pro gradu)

No. 29 2005 VAURAMO SAARA: Decomposition of chitinase transgenic silver birch (Betula pendula) leaf litter and effects on decomposer populations in a field trial (pro gradu)

No. 30 2005 IKONEN SUVI: Kasvukauden aikaiset muutokset ravinnonkäytössä kahdessa jäännehalkoisjalkaisen populaatiossa (pro gradu)

No. 31 2005 PARTANEN PASI: Bakteeridiversiteetin määrittäminen molekyylibiologisin menetelmin kompostointiprosessin eri vaiheissa (pro gradu)

No. 32 2005 MÄNTYLÄ MINNA: Humuskuormituksen vaikutus nuottaruohon (Lobelia Dortmanna L.) fotosynteesiaktiivisuuteen sekä sedimentin happioloihin ja bakteerien entsyymiaktiivisuuksiin (pro gradu)

No. 33 2005 TAIPALE TIINA: Lahopuu luonnonmetsäsukkessiossa (pro gradu)

No. 34 2006 VALENTINI MARCO: The assessment of toxicity in lead contaminated soils (master science thesis)

No. 35 2005 AALTONEN HERMANNI: Kohonneiden alailmakehän otsoni- ja hiilidioksidipitoisuuksien vaikutukset maaperän mikrobiyhteisön rakenteeseen (pro gradu)

No .36 2006 ENBERG EIJA: Ympäristö- ja laatujärjestelmän liittäminen yhteiseksi toimintajärjestelmäksi julkisen hallinnon organisaatiossa, Kymenlaakson sairaanhoitopiirin kuntayhtymässä (pro gradu)

No. 37 2006 NIITTYMIES HEIDI: Ympäristövaikutukset ja –vastuu: esimerkkinä suolakyllästämön alue (pro gradu)

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resurssikilpailu – esimerkkeinä typpi ja fosfori (pro gradu)

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No. 63 2008 HEIDI KONTIO: Lyijyllä saastuneen maaperän mikroympäristöt lyijylle herkkien bakteerilajien runsauden selittäjinä (pro gradu)

No. 64 2008 JENNI OJALA: Tuhkalla tehostetun kompostin kypsyyden ja laadun arviointi (pro gradu)